PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Coppens et al.

Serial No.: 08/898,736

Filed: July 23, 1997

Title: PROCESS FOR THE PREPARATION OF MALTED CEREALS

Group Art Unit: 1761

Examiner: C. Sherrer

CERTIFICATE OF MAILING

I hereby certify that this paper is deposited with the U.S. Postal Service as first-class mail in an envelope addressed to: Commissioner of Patents & Trademarks, Washington, D.C. 20231, on this date.

10/3/01
Date

/Timothy E Luetke/
Registration No. 30,192
Attorney for Applicants

DECLARATION RE AVAILABILITY OF MICROORGANISM AT ATCC

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

The undersigned attorney certifies that the following microorganisms (195 listed below) which are described in the claims of the above-identified application were on deposit with the American Type Culture Collection at the time the above-identified application was filed.

Micrococcus spp., Streptococcus spp., Leuconostoc spp., Pediococcus spp., Pediococcus halophilus, Pediococcus cerevisiae, Pediococcus damnosus, Pediococcus parvulus, Pediococcus soyae, Lactococcus spp., Lactobacillus spp., Lactobacillus acidophilus, Lactobacillus amyllovorus, Lactobacillus bifermentans, Lactobacillus brevis var lindneri, Lactobacillus casei var casei, Lactobacillus delbrueckii, Lactobacillus delbrueckii var lactis, Lactobacillus delbrueckii var bulgaricus, Lactobacillus fermenti,

Lactobacillus gasserii, Lactobacillus helveticus, Lactobacillus hilgardii, Lactobacillus sake, Lactobacillus kefir, Lactobacillus pentoceticus, Lactobacillus cellobiosus, Lactobacillus buchneriik, Lactobacillus coryneformis, Lactobacillus confusus, Lactobacillus viridescens, Corynebacterium spp., Propionibacterium spp., Bifidobacterium spp., Streptomyces spp., Bacillus spp., Sporolactobacillus spp., Acetobacter spp., Agrobacterium spp., Alcaligenes spp., Pseudomonas cocovenenans, Pseudomonas pseudomallei, Gluconobacter spp., Enterobacter spp., Erwinia spp., Klebsiella spp., Proteus, spp., Mycosphaerella spp., Venturia spp., Monascus spp., Emericilla spp., Euroteum spp., Eupenicillilum spp., Neosartorya spp., Talaromyces spp., Hypocrea spp., Dipodascus spp., Galactomyces spp., Endomyces spp., Metschnikowiaceae, Guilliermondella spp., Debaryomyces spp., Dekkara spp., Pichiia spp., Kluyveromyces spp., Saccharomyces spp., Torulaspora spp., Zygosaccharomyces spp., Hanseniaspora spp., Schizosaccharomyces spp., Chaetomium spp., Neurospora spp., Absidia spp., Amylomyces spp., Rhizomucor spp., Actinomucor spp., Thermomucor spp., Chlamydomucor spp., Mucor spp., Mucor circinelloides, Mucor grisecyanus, Mucor hiemalies, Mucor indicus, Mucor mucedo, Mucor piriformis, Mucor plumbeus, Mucor pusillus, Mucor silvaticus, Mucor javanicus, Mucor racemosus, Mucor rouxianus, Mucor rouxil, Mucor aromaticus, Mucor flavus, Mucor miehel, Rhizopus spp., Rhizopus arrhizus, Rhizopus oligosporus, Rhizopus oryzae, Rhizopus oryaze strain ATCC 4858, oryzae strain ATCC 9363, Rhizopus oryzae strain NRRL 1891, Rhizopus oryzae strain NRRL 1472, Rhizopus stolonifer, Rhizopus thailandensis, Rhizopus formosaensis, Rhizopus chinensis, Rhizopus cohnii, Rhizopus japonicus, Rhizopus deiemar, Rhizopus acetorinus, Rhizopus chlamydosporus, Rhizopus circinans, Rhizopus

javanicus, Rhizopus peka, Rhizopus saito, Rhizopus tritici, Rhizopus niveus, Rhizopus microsporus, Aureobasidium spp., Acremonium spp., Cercospora spp., Epicoccum spp., Monillia spp., Monillia candida, Mycoderma spp., Candida spp., Candida diddensiae, Candida edax, Candida etchellsii, Candida kefir, Candida krisei, Candida lambica, Candida melinil, Candida milleri, Candida mycoderma, Candida parapsilosis, Candida obtux, Candida tropicalis, Candida valida, Candida versatilis, Candida guillermondii, Rhodotorula spp., Torulopsis spp., Geotrichum spp., Geotrichum amycellium, Geotrichum armillariae, Geotrichum asteroides, Geotrichum bipunctatum, Geotrichum dulcitum, Geotrichum eriense, Geotrichum fici, Geotrichum flavo-brunneum, Geotrichum fragrans, Geotrichumgracile, Geotrichum penicillatum, Geotrichum hirtum, Geotrichum pseudocandidum, Geotrichum rectangulatum, Geotrichum loubieri, Geotrichum microsporum, Cladosporium spp., Trichoderma spp., Trichoderma hamatum, Trichoderma harzianum, Trichoderma koningli, Trichoderma pseudokoninglii, Trichoderma reesei, Trichoderma virgatum, Trichoderma viride, Oidium spp., Altermaria spp., Altermaria alternata, Altermaria tenuis, Helminthosporium spp., Helminthosporium gramineum, Helminthosporium sativum, Helminthosporium teres, Aspergillus spp., Aspergillus ochraseus, Aspergillus nidulans, Aspergillus versicolor, Aspergillus wentii Group, Aspergillus candidus, Aspergillus flavus, Aspergillus niger, Aspergillus oryzae strain ATCC 14156, Penicillum spp., Penicillum aculeatum, Penicillum citrinum, Penicillum claviforme, Penicillum funiculosum, Penicillum italicum, Penicillum lanoso-viride, and Penicillum liiacinum.

Copies of on-line catalogue pages showing that each microorganism is available from ATCC are attached.

Respectfully submitted,

FITCH, EVEN, TABIN & FLANNERY

By: 
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PATENT
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NO. 68897

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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draft for your 2 pat. interview

SUPPLEMENTAL DECLARATION OF THEO COPPENS UNDER 37 CFR 1.132

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

I, Theo Coppens, pursuant to 37 C.F.R. §1.132, declare as follows:

1. I am one of the inventors for the above-identified patent application.

2. In 1999, I asked Prof. C. Michiels, Professor of the Faculty of Agricultural and Applied Biological Sciences at Katholieke Universiteit Leuven in Belgium, to conduct the following experiments under my supervision to determine whether the medium and growth conditions described in Gyllang *et al.*

would provide activated spores. Those experiments and their results were first reported in my Declaration signed on July 9, 1999, and a Supplemental Declaration signed on March 3, 2000. A more detailed explanation of those experiments and their results is presented herewith.

Materials and Methods

3. Chemicals and Media. Peptone, Yeast Extract and Potato Dextrose Agar (PDA) were obtained from Unipath (Hampshire, United Kingdom). Dextrose was obtained from Merck-Belgolabo (Leuven, Belgium). Peptone, yeast extract and dextrose medium were prepared according to Kaiser et al. (1994). Peptone (2% w/v), yeast extract (1% w/v) and dextrose (2% w/v) were dissolved in deionised water. The medium was sterilized at 121°C for 15 minutes. The pH of the obtained medium was 6.4.

4. Fungal Strains: Cultivation and Preparation of Culture Homogenate. The strains *Rhizopus oryzae* ATCC 9363, *Aspergillus fumigatus* CBS 148.89 and Aspergillus *amstelodami* VTTD-76035 were obtained from respectively the American Type Culture Collection (ATCC, Manassas, VA, USA), Centraalbureau voor Schimmelcultures (CBS, Baarm, The Netherlands) and VTT (Technical Research Centre of Finland, Espoo, Finland) culture collections. The strains were grown on PDA at 28°C. Seven days old sporulating cultures on PDA served as the starting material for culturing the fungi as described by Gyllang *et al.* (1977). For each strain, a loopful of material taken from the seven-day old sporulating culture on PDA was inoculated in a tissue culture flask containing 225 ml of Peptone, Yeast Extract and Dextrose medium. The culture was grown for 3 weeks at 20°C. After the cultivation period the

entire culture was homogenized by vigorously shaking the content of the tissue culture flask.

5. Analysis of activation of the spores in the culture homogenate. Activated spores were defined as described in the current patent application as "being significantly more swollen than the dormant size, the size of the spores being increased by a factor preferably between 1.2 and 10 over the dormant spore size and/or having one or more germ tubes per spore."

One of the first steps of activation is, indeed, uptake of water, and this is reflected by an increase in volume of the spore by swelling and/or by formation of one or more germ tubes.

Three different samples of 10 μL of each culture homogenate were examined microscopically at 500X magnification (Jenaval, Hainaut, Belgium). Approximately 10 microscopic fields per sample were evaluated. To increase the amount of spores per microscopic field, the culture homogenates were concentrated by centrifugation (Hettich EBA 12, Tuttlingen, Germany; 3000 g, 5 min.) prior to microscopic evaluation.

Photomicrographs of all the microscopic fields were taken and the size of the spores was measured on the printouts of these images. This is in contrast to the method used in the experiments described in the previous report, where the spore size was evaluated directly by microscopic analysis.

Images of the microscopic fields were captured by means of a JVC (TK-C 1381) digital color video camera and saved as a windows bitmap (24 bit) using miro Television software. The image of a microscopic grid (WILD, Heerbrugg, Switzerland; 0.01 mm; 0.10 mm) was captured at the same magnification as was used to capture the images of the spores. This allowed to calculate the

magnification factor and the actual spore size from the measured size on the printouts.

All images were printed in the same manner (full scale) and the size of the spores not occurring in flocs or pairs and that were not attached to mycelium fragments was measured on the printouts. Each spore was measured in the same manner: the largest diameter was measured including the edges of the spore. The actual spore size was calculated by dividing the measured spore size on the printout by the magnification. Also, the absence or presence of germ tubes was recorded for each spore. All observed spores were divided in classes according to size, each class spanning a range of 0.8μm. This gives five size classes for *R. oryzae*, three for *A. fumigatus*, and four for *A. amstelodami*. This allows us to detect any shift in spore size that would occur between incubation time 0 hours and incubation time 6 hours, and that would be indicative of spore activation.

Results

6. Analysis of spore activation. The size of various dormant fungal spores is described by Pitt and Hocking (1997). According to this reference, the sporangiophores of *Rhizopus oryzae* are of variable shape, ellipsoidal to broadly fusiform or irregularly angular, commonly 5.0-8.0 μm long, the condiospores of *Aspergillus amstelodami* are spherical to subspheroidal with 4.0-5.0 μm diameter; the condiospores of *Aspergillus fumigatus* are spherical to subspheroidal with 2.5-3.0 μm diameter. Our own observations of dormant spores of the three tested strains according to the method described above were as follows: the sporangiophores of *Rhizopus oryzae* were 3.9-7.8 μm long; the

condiospores of *Aspergillus amstelodami* had a diameter of 3.1-6.3 μm; the condiospores of *Aspergillus fumigatus* had a diameter of 2.3-4.7 μm.

The results of the evaluation of the spore size in the culture homogenates at time 0 hours and after 6 hours incubation at 20°C or at 42°C for *Rhizopus oryzae, Aspergillus amstelodami* and *Aspergillus fumigatus* are shown in Tables I, II and III respectively.

Table I - Size Distribution of Spores Occurring in Culture Homogenates of *Rhizopus Oryzae*

Incubation time - 0 h.						
Size Range[a] (μm)	Number of Spores			% of the Total Number of Analyzed Spores		
	1[b]	2[b]	3[b]	1[b]	2[b]	3[b]
3.9 - 4.7	24	54	31	16	23	18
4.7 - 5.5	67	125	99	46	54	59
5.5 - 6.3	55	48	38	37	21	22
6.3 - 7.0	1	2	1	1	1	1
7.0 - 7.8	-	1	-	0	1	0
Total No. of Spores	147	230	169			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

[b] Three samples of 10 μl of spore suspension analyzed for each incubation condition.

Incubation time - 6 h., 20°C						
Size Range[a] (μm)	Number of Spores			% of the Total Number of Analyzed Spores		
	1[b]	2[b]	3[b]	1[b]	2[b]	3[b]
3.9 - 4.7	43	74	35	26	43	24
4.7 - 5.5	87	72	67	52	42	45
5.5 - 6.3	32	26	45	19	15	30
6.3 - 7.0	3	-	2	2	0	1
7.0 - 7.8	1	-	-	1	0	0
Total No. of Spores	166	100	172			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

[b] Three samples of 10 μl of spore suspension analyzed for each incubation condition.

Incubation time - 6 h., 42°C						
Size Range[a] (μm)	Number of Spores			% of the Total Number of Analyzed Spores		
	1[b]	2[b]	3[b]	1[b]	2[b]	3[b]
3.9 - 4.7	30	19	20	10	14	20
4.7 - 5.5	105	58	54	36	41	53
5.5 - 6.3	142	62	26	49	44	25
6.3 - 7.0	10	1	2	4	1	2
7.0 - 7.8	2	-	-	1	0	0
Total No. of Spores	289	140	102			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

[b] Three samples of 10 μl of spore suspension analyzed for each incubation condition.

Table II - Size Distribution of Spores Occurring in Culture Homogenates of *Aspergillus fumigatus*

Time - 0						
Range[a] (μm)	Number of Spores			% of the Total Number of Spores		
	1	2	3	1	2	3
2.3 - 3.1	27	20	22	22	18	16
3.1 - 3.9	67	71	79	56	64	58
3.9 - 4.7	26	20	35	22	18	26
Total No. of Spores	120	111	136			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

Time - 6 h., 20°C						
Range[a] (μm)	Number of Spores			% of the Total Number of Spores		
	1	2	3	1	2	3
2.3 - 3.1	10	8	7	9	6	6
3.1 - 3.9	65	112	66	61	89	59
3.9 - 4.7	32	6	39	30	5	35
Total No. of Spores	107	126	112			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

Time - 6 h., 42°C						
Range[a] (μm)	Number of Spores			% of the Total Number of Spores		
	1	2	3	1	2	3
2.3 - 3.1	6	3	11	4	3	8
3.1 - 3.9	84	68	96	56	72	66
3.9 - 4.7	61	24	39	40	25	26
Total No. of Spores	151	95	148			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

Table III - Size Distribution of Spores Occurring in Culture Homogenates of *Aspergillus amstelodami*

Time - 0						
Range[a] (μm)	Number of Spores			% of the Total Number of Spores		
	1	2	3	1	2	3
3.1 - 3.9	10	4	7	10	4	6
3.9 - 4.7	52	54	64	51	52	56
4.7 - 5.5	30	34	31	29	32	27
5.5 - 6.3	10	13	13	10	12	11
Total No. of Spores	102	105	115			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

Time - 6 h., 20°C						
Range[a] (μm)	Number of Spores			% of the Total Number of Spores		
	1	2	3	1	2	3
3.1 - 3.9	2	-	2	3	0	5
3.9 - 4.7	44	17	21	67	41	51
4.7 - 5.5	14	19	15	21	45	37
5.5 - 6.3	6	6	3	9	14	7
Total No. of Spores	66	42	41			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

Time - 6 h., 42°C						
Range[a] (μm)	Number of Spores			% of the Total Number of Spores		
	1	2	3	1	2	3
3.1 - 3.9	2	-	-	3	0	0
3.9 - 4.7	37	32	37	61	73	53
4.7 - 5.5	17	10	21	28	23	30
5.5 - 6.3	5	2	12	6	4	17
Total No. of Spores	61	44	70			

[a] The size of a spore in a certain range is larger than the lower limit and smaller than or equal to the upper limit.

The results show no shift towards a larger spore size. This means that, for none of the three fungi studied, the spores increase in size when spore suspensions are prepared according to Gyllang et al. In addition, none of the observed spores had developed a germination tube.

Spore activation is not a process that occurs quickly and if spore activation occurred at 0 hours, one would see further activation at least after 3 hours, and would certainly see further activation after 6 hours.

In contrast, treatment of *Rhizopus oryzae* ATCC 9363 spores as described in the current patent application resulted in a high level of activation of the spores as more than 90% of the spores had a size of more 9.4 μm and/or had one or more germ tubes per spore, hence, providing further evidence of non-activation of spores as described in the present data.

7. Conclusions. Culture homogenates of *Rhizopus oryzae* ATCC 9363, *Aspergillus fumigatus* CBS 148.89 and *Aspergillus amstelodami* VTT D-76035 prepared according to Gyllang *et al.* (1977) do not contain activated spores.

This experiment shows that successful activation depends on incubation of dormant spores for a sufficient time at a suitable temperature and in a suitable medium. In the spore suspension as prepared by Gyllang *et al.* (1977), the medium is an exhausted growth medium that does not provide the suitable conditions for spore activation, and the spores are not incubated for a sufficient time at a sufficient temperature.

8. Abbreviations used. PDA, Potato Dextrose Agar; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; VTT, Technical Research Centre of Finland; Ac, activated.

9. References.

- Gyllang, H., Sätmark, L. and Martinson, E., The influence of some fungi on malt quality, *EBC. Proceedings of the 16th Congress,* 1977.
- Kaiser, C., Michaelis, S. and Michell, A., *Methods in yeast genetics,* Appendix A, p. 207, Cold Spring Harbor Laboratory Press, New York, USA, 1994.
- Pitt, J.I. and Hocking, A.D. Fungi and food spoilage, second edition, Blackie Academic & Professional, London, UK, 1997.

10. Addendum. The printed photomicrographs (40% relative to the original image size) of sample 1 of each experimental condition are included in Figures 2 to 10. The printed photomicrograph (40% relative to the original image size) of the microscopic grid is included in Figure 1. Al original images are included in digital format (CD-ROM).

Figure 1	microscopic grid;
Figure 2	*Rhizopus oryzae* ATCC 9363, spores in the culture homogenate at time 0 (sample 1);
Figure 3	*Rhizopus oryzae* ATCC 9363, spores in the culture homogenate after 6 hours incubation at 20°C (sample 1);
Figure 4	*Rhizopus oryzae* ATCC 9363, spores in the culture homogenate after 6 hours incubation at 42°C (sample 1);
Figure 5	*Aspergillus fumigatus* CBS 148.89, spores in the culture homogenate at time 0 (sample 1);

Figure 6	*Aspergillus fumigatus* CBS 148,89, spores in the culture homogenate after 6 hours incubation at 20°C (sample 1);
Figure 7	*Aspergillus fumigatus* CBS 148.89, spores in the culture homogenate after 6 hours incubation at 42°C (sample 1);
Figure 8	*Aspergillus amstelodami* VTT D-76035, spores in the culture homogenate at time 0 (sample 1);
Figure 9	*Aspergillus amstelodami* VTT D-76035, spores in the culture homogenate after 6 hours incubation at 20°C (sample 1);
Figure 10	*Aspergillus amstelodami* VTT D-76035, spores in the culture homogenate after 6 hours incubation at 42°C (sample 1).

The undersigned, being warned that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon, hereby declares that the above statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date:_____ _____

Theo Coppens